Biochimica et Biophysica Acta 1798 (2010) 958–965

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Temperature-induced structural transition in-situ in porcine lens — Changes observed in void size distribution

Petri Sane [a], Filip Tuomisto [a], Susanne K. Wiedmer [b], Tuula Nyman [c], Ilpo Vattulainen [a,d,e], Juha M. Holopainen [f,*]

[a] Department of Applied Physics, Aalto University, Finland
[b] Laboratory of Analytical Chemistry, Department of Chemistry, University of Helsinki, Finland
[c] Protein Chemistry Research Group and Core Facility, Institute of Biotechnology, University of Helsinki, Finland
[d] Department of Physics, Tampere University of Technology, Finland
[e] Memphys – Center for Biomembrane Physics, University of Southern Denmark, Denmark
[f] Helsinki Eye Lab, Department of Ophthalmology, University of Helsinki, Haartmaninkatu 4 C, 00290 Helsinki, Finland

ARTICLE INFO

Article history:
Received 18 October 2009
Received in revised form 3 January 2010
Accepted 14 January 2010
Available online 1 February 2010

Keywords:
Lens
Lipid membranes
Positron annihilation lifetime spectroscopy
Differential scanning calorimetry
Sphingomyelin

ABSTRACT

The function of mammalian ocular lens is to provide a sharp image to the retina. Accordingly, the lens needs to be transparent and minimize light scattering. To do so the lens fiber cells first loose intracellular organelles, organize the cytoplasm and arrange the fiber cell membranes. Because the fiber cells are metabolically inactive, the plasma membrane becomes the only cellular organelle and consequently, the phase behavior of these membranes determines the physiological state of the lens. Previous studies have shown that lipids extracted from the nuclear and cortical region of human lens show a temperature-induced phase transition close to the body temperature. Yet, the physiological function of this phase transition is not known, and even the presence of the phase transition in intact lenses is unknown. Positron annihilation lifetime spectroscopy (PALS) was used to characterize the sub-nanometer-sized local structure of intact porcine lens and these studies were complemented with differential scanning calorimeter and mass spectrometric analysis in extracted porcine lens lipids. Using PALS, we present evidence for the presence of a temperature-dependent structural transition centered at 35.5 °C in-situ in clear extracted porcine lenses. Further studies employing extracted lens lipids and purified egg-yolk sphingomyelin and cholesterol mixtures suggest that the nano-scale transition emerges from the phase behavior of lens lipids. Based on our results, PALS seems to be a viable method for gaining additional information on biological tissues, especially since it enables non-destructive studies on intact tissues.

1. Introduction

The avascular intraocular lens is composed of concentric layers of enucleated fiber cells. The newly formed fiber cells are translocated from the equatorial plane of the lens towards the lens center. During this progressive displacement, these cells lose their internal organelles and the cells are layered on top of each other, forming an onion-like structure where the oldest fiber cells are positioned in the innermost segment of the lens and the newer ones are found in the cortical region of the lens. This process begins early in embryogenesis and continues throughout life. Consequently during aging the lens weight increases and the rigidity of the lens increases towards the nucleus. The latter effect is believed to be due to the unique lipid composition of the lens [1–4].

During the translocation process fiber cells lose their internal organelles by a lipoxygenase regulated mechanism [5]. As a result, the plasma membrane becomes virtually the only membrane of the lens. In order to preserve transparency and minimize light scattering, these membranes need to be highly ordered. The cytoplasm of fiber cells is largely composed of crystallin proteins composed of two gene families: α- and βγ-crystallins [6]. It seems that the α-crystallins are molecular chaperones preserving lens clarity by preventing protein aggregation [7]. The plasma membrane is unique in that it contains high concentrations of the protein aquaporin 0 (AQP0), a 26-kDa water channel, lacks polyunsaturated phospholipids, and has very high concentrations of sphingomyelin (SM) and cholesterol [8,9]. In fact, the cholesterol content of the lens is the highest among all tissues. What is more, the cholesterol content increases even further as the fiber cells mature [10,11]. The presence of high concentrations of SM and cholesterol are likely to increase the rigidity of the fiber cell plasma membranes. Borchman and colleagues have shown that lipid membranes isolated from the cortical and nuclear fiber cell plasma membranes display a temperature-dependent phase transition close

* Corresponding author. Tel.: +358 9 4717 7197.
E-mail address: juha.holopainen@hus.fi (J.M. Holopainen).

0005-2736/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2010.01.011

to the body temperature and close to the main phase transition temperature of SMs [12–15]. Later Borchman et al. showed that in lipid membranes isolated from cataractous lenses this phase transition temperature is higher than in clear lenses [15]. Yet, it remains unclear whether similar phase transitions could be found in intact lenses or are they just artifacts resulting from lipid isolation. Furthermore, the physiological function of this phase transition remains an open issue, and equally unclear is whether such phase transition could play a role in cataractogenesis.

Positron annihilation lifetime spectroscopy (PALS) is a well-established and very sensitive technique for probing sub-nanometer-sized local free volumes in molecular solids [16–18]. In addition, positron emission tomography (PET) [19,20] has found insightful applications as a diagnostic technique in e.g. clinical oncology and neuroimaging. Accordingly, the monitoring of positron annihilation signals through β^+-active radiotracers is applied to locate tumors and the blood flow for instance in neural tissues. We have recently demonstrated the applicability of PALS to study temperature-dependent effects in biological materials [21]. Importantly, with this technique it is possible to study phase transitions and changes in free volume of lipid bilayers in-situ.

Here we have studied the free volume changes and possible nanoscale structural transitions in-situ in intact porcine lenses using PALS. In essence, we show that within a narrow temperature range the free volume distribution is changed drastically, most likely representing a temperature-induced structural transition in intact porcine lens.

2. Materials and methods

Porcine eyes were obtained from a local abattoir and transferred to the laboratory within 6 h of death. The lenses were extracted immediately after transfer by excision from an anterior approach, and the lens weight was recorded. The intact lenses were either used for PALS measurements (see below) or the lipids were extracted by the Folch method [22]. In brief, for lipid extraction whole lenses were homogenized in 6 ml phosphate buffered saline (pH 7.4) at room temperature using a glass homogenizer. Total lipids were then extracted from this material using 10 volumes of chloroform:methanol (2:1, vol/vol). The lower organic phase was separated and the organic solvents were evaporated under nitrogen. The dried lipids were weighted and dissolved into chloroform to yield a final total lipid concentration of 1 mg/ml.

Egg-yolk sphingomyelin (eggSM) and cholesterol were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. Concentrations of the lipids were determined gravimetrically and the purity of the above lipid was verified using thin layer chromatography. Water was freshly deionized with a Milli RO/Milli Q (Millipore, Bedford, MA) filtering system.

1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleyl-*sn*-glysero-3-phosphor-L-serine (POPS), and N-hexadecanoyl-D-erythro-ceramide (C16-ceramide) were obtained from Avanti Polar Lipids (Alabaster, AL).

2.1. Positron annihilation lifetime spectroscopy

2.1.1. Background

When an energetic positron from a radioactive source (^{22}Na in most cases) enters molecular media, it thermalizes in a very short time after interacting with the surrounding molecules through inelastic collisions. A fraction of positrons forms a bound state with spin parallel electrons called the ortho-positronium (o-Ps) that preferentially localize in the free volume pockets in the material. In vacuum, o-Ps has a relatively long lifetime of 142 ns, compared to 125 ps for p-Ps (bound state of a positron and an electron with opposite spins). In a medium, the o-Ps prefer to undergo so called pick-off annihilation with an electron of opposite spin during collision with molecules in the cavity wall in which they are localized. The pick-off process reduces the lifetime of o-Ps from 142 ns to a few ns. Accordingly, *the smaller the cavity size, the higher the frequency of collisions and the shorter the o-Ps lifetime*, thus providing information of the size of the free volume pockets (i.e. voids).

The positron/positronium lifetime is measured with a pair of scintillation detectors, one detecting the birth of a positron and the other its annihilation. When the positron source (^{22}Na) decays, it produces simultaneously one positron and one ~1.27 MeV γ-quantum. When this quantum denoting the birth of the positron is detected, the pulse acts as a START-signal for the equipment. When the positron annihilates in the sample, as positron, p-Ps or o-Ps (via pick-off), it produces two γ-quanta of about 511 keV each, either of which acts as the STOP-signal. The time difference between the START- and STOP-pulses is a measure of the positron lifetime in the material [23].

Almost all biological material contains water and hence some of the o-Ps's always annihilate in water. Because the lifetime component of the biomaterial is typically quite near the temperature-independent water lifetime, the measured lifetime component is often an intensity weighted sum of water and biomaterial lifetimes. The summed lifetime corresponds strongly to the weight% of water in the sample, causing the lifetimes measured in dilute solutes to be near the lifetime measured in pure water (~1.8 ns) and also the changes caused by adding small amounts of lipid membranes are small in the summed lifetimes.

Due to the deterioration of biological samples, detection efficiency is vital for gaining maximum amount of information e.g. as many temperature steps as possible. Therefore we have decided to use detectors with large physical dimension, for the cost of absolute resolution. The difference in a typical DPPC sample between two typical sized scintillation detectors is shown in Fig. 1 [23].

Even though the loss of resolution makes separation of changes in individual lifetime components more difficult due to the strong effect of o-Ps atoms annihilating in water, increased statistics improve the reliability of the results. Accordingly, when change is observed in the "summed lifetime", it is most likely a real physical change occurring in the sample.

2.2. Experimental

Altogether six individual porcine lenses were studied. Before the source injection, excess saline was removed from the test tube leaving the upmost surface of the lens peep out under the saline surface, thus the amount of non-active saline left inside the test tube varied in each sample. ^{22}Na positron source material was injected as aqueous

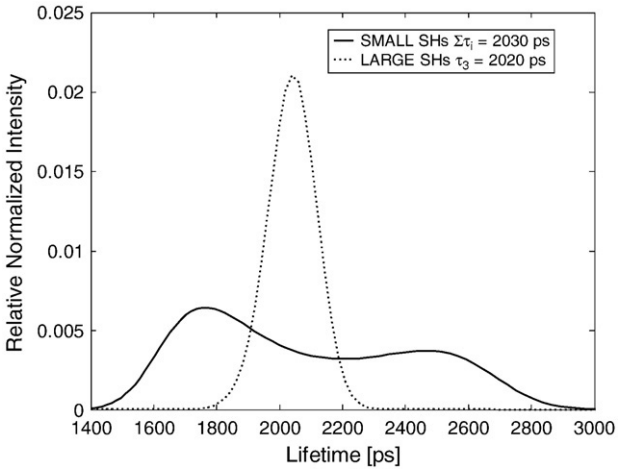

Fig. 1. Plots of lifetime distributions in lipid bilayers (DPPC) in aqueous solution measured separately with small (solid line) and large (dotted line) scintillation heads. τ_3 being the measured lifetime with large scintillation heads, whereas $\Sigma\tau_3$ denotes the intensity weighted sum of measured lifetimes in water and lipid material.

solution (saline) directly inside the lens with a 30 gauge needle, causing minimal damage to the lens. The concentration of NaCl injected to the sample was calculated to be less than 0.02 μM, which does not have any significant effect on the properties of the sample. After the source injection the test tube was sealed and inserted into the temperature controlling setup between the detector pair.

Typical activity of the injection was approximately 2 MBq (compared to several hundred MBq used in PET [20]), causing roughly 5 Sv equivalent radiation dose to the lens during the average ~24 h measurement cycle. The dose is reasonably high, 30 times higher than the recommended dose for human lens [24]. However, the mean positron energy of 180 keV is not high enough for severe microstructural damage that would skew the results during the measurement period. In earlier measurements [21], no effect of radiation damage in the o-Ps lifetimes has been observed for lipid membranes within the 24 h measurement period.

To prepare multilamellar vesicles for PALS studies, two sets of experiments were carried out. In the first set, lipids from 10 clear porcine lenses were extracted as described above, dried and used for vesicle preparation. In the second set, 250 mg of pure eggSM or mixtures of eggSM with cholesterol were weighted into an Eppendorf tube. For both lipid samples the dry lipid was hydrated with 0.5 ml of buffer (20 mM HEPES, 0.1 mM EDTA, 150 mM NaCl, pH of 7.4) at 80 °C to yield multilamellar vesicles, and the vesicles were maintained at this temperature for 60 min with subsequent shaking. During hydration, the vesicle-containing solution was vortexed four times.

Temperature of all the PALS samples was controlled with a commercial temperature controller, keeping the temperature variation within 0.05 °C during each measurement step. Because the sample lenses were kept in closed test tubes, direct measurement of the sample temperature was not possible; therefore the temperature inside the sample was estimated from calibration data obtained through extensive thermal testing prior to actual measurements, with an error less than 0.2 °C compared to the nominal sample temperature. Thus, the actual sample temperatures for each sample in the same nominal temperature step are not accurate, however within individual measurement series, the temperature difference between steps was the fixed step difference with less than 0.05 °C of error.

2.3. Differential scanning calorimetry

For differential scanning calorimetry (DSC) experiments, 1 mg of extracted total lens lipids in chloroform was transferred to a glass vial and then evaporated to dryness under a stream of nitrogen. Traces of solvent were removed by evacuating under reduced pressure for 24 h. The lipid residues were hydrated at 80 °C for 30 min in 1 ml of buffer (20 mM HEPES, 0.1 mM EDTA, 150 mM NaCl, pH of 7.4) to yield multilamellar vesicles.

EggSM or eggSM–cholesterol multilamellar vesicles were prepared as above.

Differential heat capacity scans were recorded at a lipid concentration of 1 mg/ml for extracted lens lipids and 1 mM for eggSM and eggSM–cholesterol vesicles at a heating rate of 0.5 °C/min. Before their loading into pre-cooled DSC cuvettes, the samples were equilibrated on ice for ~24 h and thereafter degassed at low pressure. The calorimeter (VP-DSC, MicroCal, Northampton, MA) was interfaced to a PC and data were analyzed using the routines of the software provided with the instrument. All samples were scanned twice by heating from 10 to 80 °C followed by cooling from 80 to 10 °C. DSC experiments were done in triplicates.

2.4. Phospholipid analysis by matrix-assisted laser desorption ionization–time of flight mass spectrometry

The porcine lens lipid sample (in chloroform) was mixed 1:4 with the matrix (150 mM p-nitroaniline in chloroform/methanol (2:1% v/v), with crystals of cesium chloride [25]. Mass spectra were acquired using an Ultraflex TOF/TOF instrument (Bruker-Daltonik GmbH, Bremen, Germany) equipped with an 337 nm UV nitrogen laser in a positive ion reflector mode. The instrument was externally calibrated using a standard mixture of peptides from Bruker. The lipid ionization parameters were checked with synthetic lipid standards (POPC, POPS, DMPC, and C16 ceramide).

3. Results

3.1. Positron annihilation lifetime spectroscopy in-situ in intact porcine lenses

PALS studies for each lens were performed as a series of measurements consisting of individual temperature steps, in which the positron lifetime spectrum was measured. Based on the results of preliminary PALS measurements which showed anomalous behavior at ~35 °C, the temperature range from 33 to 36 °C was studied more carefully. For the first two lenses, we used a temperature step of 0.5 °C, while for lenses #3–6 the temperature dependence was studied more carefully in terms of a temperature step of 0.3 °C.

To improve statistics, we gathered millions of annihilation events to ensure that the conclusions will be on a solid ground. This is emphasized by the fact that only a fraction of occurring lifetime events are actually detected, and most of them (~75%) are caused by annihilating positrons that have not formed an ortho-positronium (o-Ps), thus the yield measured in our experiments is not optimal. To overcome these limitations, we extended measurement times to make sure that at least two million lifetime events are measured for each individual spectrum. From a typical case, three lifetime components were evident, the longest one of them (τ_3) being caused by o-Ps's annihilating via pick-off process within the lens [16]. The lifetime component τ_1 represents the lifetime of free positrons and p-Ps, and τ_2 is associated with the shorter o-Ps lifetime component.

The conditions for conducting measurements are not ideal since the nominal sample temperature fluctuates to some extent within each sample, and the amount of saline inside the test tube may also vary. These uncertainties create minor differences in the o-Ps lifetimes between different lenses at specific nominal temperatures. Consequently, since the lifetime data for lenses #1–#5 were in agreement, all behaving in a similar manner, we present their average behavior in Fig. 2. For the lens #6, no temperature dependence was found. While

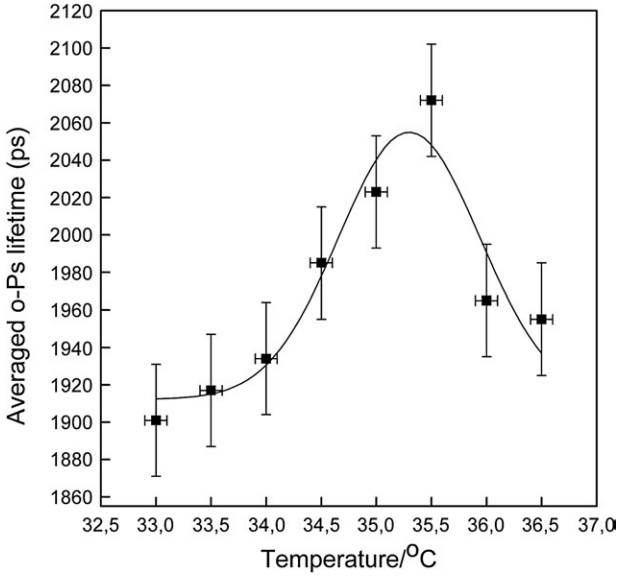

Fig. 2. o-Ps lifetimes (τ_3) averaged over data for lens #1–5. A temperature-induced anomalous transition is observed between 34.5 and 35.5 °C.

the reason for this remains unclear, it is likely that this is related to air bubbles in the sample.

The data in Fig. 2 shows that the o-Ps lifetime component exhibits a clear peak around 35.5 °C, reflecting an increase in the average size of free volume (voids) in the lens. Here, to better understand the reasoning associated with this finding, there is reason to stress that the larger is the free volume in a pocket, the less likely is that the positronium will annihilate. Hence, long lifetimes correlate with large free volume pockets.

3.2. Positron annihilation lifetime spectroscopy of lens lipids and egg-yolk sphingomyelin multilamellar vesicles

The anomalous behavior observed above with PALS in-situ in intact porcine lenses could basically result from physical changes in either the lens proteins or lens lipids. Because the main lens proteins are not known to show temperature-dependent phase transition close to 40 °C, the most likely candidate would then be the lens lipids. Since sphingomyelins are known to exhibit a main phase transition at ~40 °C [12,13] it seemed reasonable to assume that the change in the lens free volume would be caused by a phase transition of this lipid. To gain better understanding of the origin of the anomalous behavior of intact lenses, two additional lipid samples were examined by PALS: lipids separated from lenses and pure eggSM and eggSM–cholesterol multilamellar vesicles. To begin with, lipids from porcine lenses were extracted by the Folch method [22]. Because of low yield, we were only able to prepare samples of low concentration in aqueous solution. By doing so, the impact of o-Ps annihilation in the lipid regions was reduced compared to that taking place in water. The high concentration of water caused the changes in o-Ps lifetime to be smaller than in lens samples, but the same lifetime peak observed in lens samples was also observed in extracted lens lipids (Fig. 3).

EggSM multilamellar vesicles were formed in buffer as described in Materials and methods. To obtain a high signal-to-noise ratio, a very high lipid content (50 wt.%) was used. After positron source injection, the test tubes were sealed. Temperature scan from 23 °C to 61 °C was performed in steps of 2 °C, performing the scan twice, upwards and downwards. Additional measurements were also performed close to 40 °C, where the main phase transition of eggSM occurs (see the DSC results below and Ref. [9]). The results show a very clear main phase transition for eggSM at ~40 °C (Fig. 3 insert).

Since the lens is known to contain high concentrations of cholesterol, additional experiments using a mixture of eggSM with cholesterol were carried out. Two additional samples were studied that contained 15 and 30 mol% of cholesterol. Fig. 3 shows the temperature dependency of o-Ps lifetime as a function of temperature. It essentially shows that both lipid mixtures display a peak-like increase in the o-Ps lifetime at ~35–36°. Furthermore, at 30 mol% of cholesterol the peak in the o-Ps lifetime is wider than that in the 15 mol% cholesterol sample, in agreement with the lens lipid sample. As expected due to steric reasons the o-Ps lifetime is significantly shorter in the samples containing cholesterol compared to pure eggSM bilayers.

3.3. Differential scanning calorimetry

DSC was used to detect possible phase transitions in extracted lens lipids and also in eggSM multilamellar vesicles. Representative DSC up- and downscans for lens lipids are presented in Fig. 4. For the lens sample the first upscan from 10 to 80 °C did not reveal any discernible endo- or exotherms. Likewise, the first downscan showed only a flat, monotonous DSC trace. Further up- or downscans did not induce any detectable change in the heat capacity curve (Fig. 4).

In contrast to the above, for eggSM vesicles we found a very clear phase transition centered at 38.9 °C during upscan (Fig. 5). This transition seemed reversible as it was also observed in downscans, where the main phase transition temperature was lowered only slightly to 38.4 °C. These results are in good accordance with those from Epand [12]. Finally, two additional samples containing a mixture of eggSM and cholesterol were studied. Including 15 mol% of cholesterol into eggSM vesicles divided the endothermic peak into two peaks, centered at 37.5 and 39.7 °C (Fig. 5). These peaks were practically reversible upon cooling. Further increase in cholesterol content to 30 mol% nearly abolished the temperature-dependent

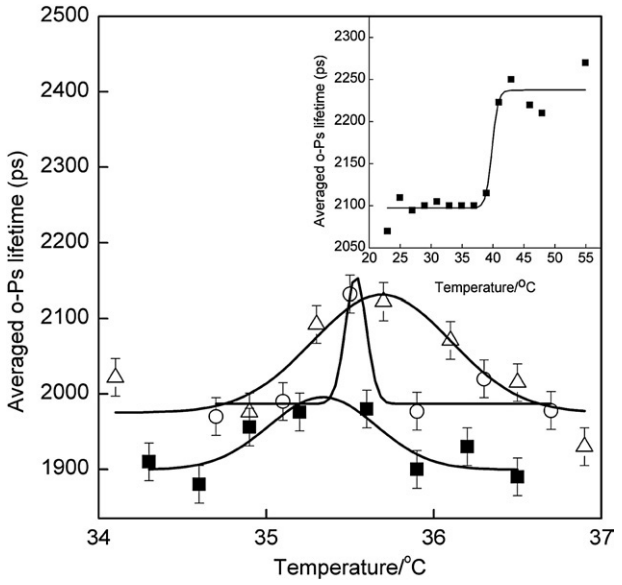

Fig. 3. Measured o-Ps lifetimes (τ_3) in lipids separated from mammalian lenses (■), and measured lifetimes in a eggSM–cholesterol mixtures containing 15 (○) or 30 (△) mol% of cholesterol. The insert shows averaged lifetimes in eggSM multilamellar vesicles (50/50 wt.%) presenting the clear main phase transition occurring in SM. Note that the behavior of the lifetime change in lens lipids and eggSM/cholesterol mixtures is similar to that observed in intact lenses, see Fig. 2. The absolute lifetimes cannot be compared to each other due to the low lipid yield in separated lipids.

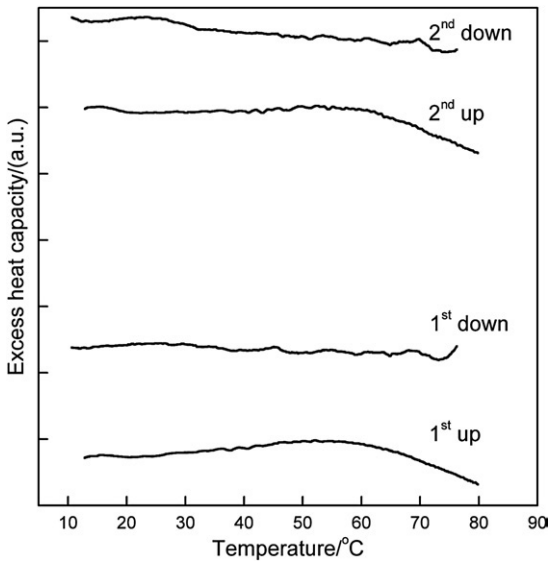

Fig. 4. Representative DSC analysis of extracted porcine lens lipids. The lipid concentration was 1 mg/ml in 20 mM HEPES, 0.1 mM EDTA, 150 mM NaCl, pH of 7.4. The figure shows two subsequent heating–cooling scans at a rate of 0.5 °C/min.

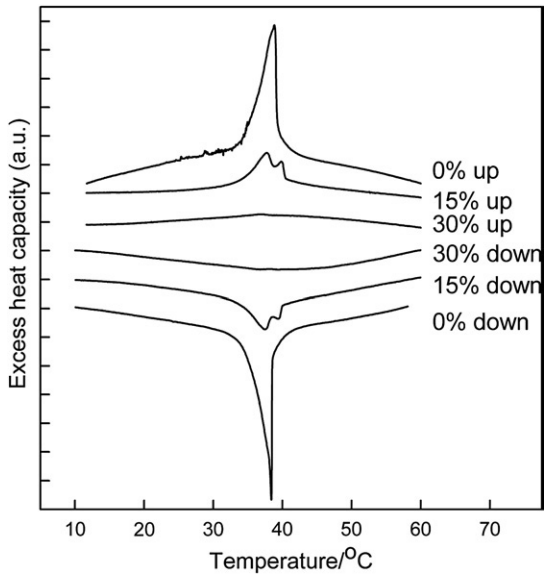

Fig. 5. Representative DSC up- and downscans for purified eggSM and eggSM/cholesterol (mol% of cholesterol is given in the graph) multilamellar vesicles. The lipid concentration was 1 mM in 20 mM HEPES, 0.1 mM EDTA, 150 mM NaCl, pH of 7.4. The figure shows one heating (trace a) and cooling (trace b) scan. The heating/cooling rate was 0.5 °C/min.

change in DSC traces. Yet, a very wide peak centered at ~38 °C was observed in both up- and downscans.

3.4. Matrix-assisted laser desorption ionization time of flight mass spectrometry of porcine lens lipids

Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) was used to identify the major phospholipid components of porcine lens. To facilitate the viewing of the results, the MALDI-TOF MS chromatogram of porcine lens lipids is shown in Fig. 6 and Table 1. The ions were identified according to literature [25–27]. In keeping with previous findings [26], the major phospholipid components detected were long chain phosphatidylcholines (PCs) and SMs. In this setup, cholesterol could not be detected.

4. Discussion

We have previously shown [21] that there is a strong and direct correlation between the void sizes in lipid bilayers and the o-Ps lifetimes. Furthermore, we have shown both earlier [21] and also in this work that PALS is a very sensitive method to study changes in void size during temperature-induced phase transitions in lipid bilayers.

Here, we have used PALS to detect possible phase transitions in-situ in intact porcine lenses. The motivation for this study derives from the seminal work by Borchman et al. who showed that extracted lipids from human lenses showed a temperature-dependent phase transition close to the body temperature [14,15]. Our data essentially shows that at approx. 35.5 °C an anomalous behavior in the o-Ps lifetime τ_3 is observed in-situ in intact lenses. Based on purified lens lipid extract and pure lipid measurements, we suggest that the observed effect is caused by the lipids in the lens itself. This finding is in very good agreement with those from Borchman et al. who showed that the cortical lipids displayed a main phase transition at 36–39 °C [14,15]. MALDI-TOF MS analysis showed that the major phospholipid species in porcine lens are long chain SMs and PCs. EggSM and also brain derived SM possess a phase transition close to 40 °C (see Fig. 5 and ref. [28]). Accordingly, to begin with, it seemed reasonable to assume that the structural transition observed in PALS experiments in-situ in porcine lens derived from the SMs in these lenses. Yet, as the free volume characteristics result from the material properties of the lens itself, chemical reactions could also have an effect on the o-Ps lifetime. Thus it is also possible that the o-Ps lifetime not only correlates with the free volume of the lens epithelial cell lipid membranes, but furthermore it provides information on the dynamics of the biomolecular system, e.g., how the high frictional resistance the material poses to the o-Ps is rationalized as the o-Ps diffuses between the molecules. The less dense materials allow o-Ps to pass through, and thus the probability of pick-off annihilation becomes smaller for an increasing lifetime of o-Ps. Based on the theoretical work (especially the Tao-Eldrup model) on o-Ps trapping in polymers [17], it is possible to calculate a speculative estimate for the change in free volume in the observed transition. Using the minimum lifetime of 1900 ps on the base of the peak in Fig. 1 and the peak maximum of 2070 ps, it would indicate an increase of ~18% to the median volume of the free volume pockets (voids). We must, however, emphasize that the model used to calculate these values has been designed for polymer melts, more specifically for spherical voids; still, lipids are also (short) polymers, and the change of the volume can be used as an indicator of the scale of changes. Thus, change of this caliber can have a significant effect on the diffusion properties of the bilayers involved and therefore likely affect the properties of membrane embedded proteins, especially AQP0 and other intrinsic proteins [29]. In fact, Gonen et al. [30] have shown that the packing interactions between AQP0 tetramers in the crystalline array are mediated by lipid molecules. Thus, a change from a gel-like environment to a fluid-like one is expected to affect the activity of the protein.

Even if the exact means of o-Ps interactions with the structures and molecules of the biomolecular materials remain partly unknown, it is clear that the changes in the o-Ps lifetime are real rather than due to random fluctuations. The averaged data in Fig. 2 contains tens of millions of lifetime events and provide a reliable statistics for the analysis. As the increase in o-Ps lifetimes is strongly temperature related, it is most likely caused by a small change in the dynamics and free volume characteristics of the lens material. Because it is unlikely (at 35.5 °C) that the main proteins, AQP0 or crystallins, affect the o-Ps lifetime, the most likely source of the observed anomalous behavior is the lens fiber cell membrane. The change could be small and insignificant on a macrostructural level, but the nanometer-sized microstructure definitely undergoes a structural transition, which the o-Ps probe is able to gauge. Whether the enhanced free volume and the transition have physiological effects on the function of the lens, remain to be clarified.

DSC did not reveal any discernible phase transition in the studied temperature range for lens extracted lipid samples. Although this might be considered to be in contradiction to the above and to previous results from Borchman's group [14,15], the likely cause is that the enthalpy changes associated with the structural transition are so small that the sensitivity of DSC is not high enough. Furthermore, due to the lack of co-operativity in such lipid mixtures these transitions are very wide and thus make the detection of these unlikely. It should be emphasized that cooling from 80 °C did not induce phase separation in lens lipid samples. To overcome these problems with DSC, we extracted lipids from porcine lenses for analysis with PALS. Because in PALS experiments tens of milligrams of lipid are needed for good sampling, we had to accept some of the problems arising when using very dilute samples. Yet, even with these dilute lipid samples a clear temperature-dependent increase in o-Ps lifetime was seen at ~35.5 °C (Fig. 3). Because the main phase transition temperatures of eggSM (Fig. 5) and brainSM are at ~40 °C [28] and because extracted lens lipids [14,15] show a main phase transition at 36–39 °C, it was feasible to assume that the observed

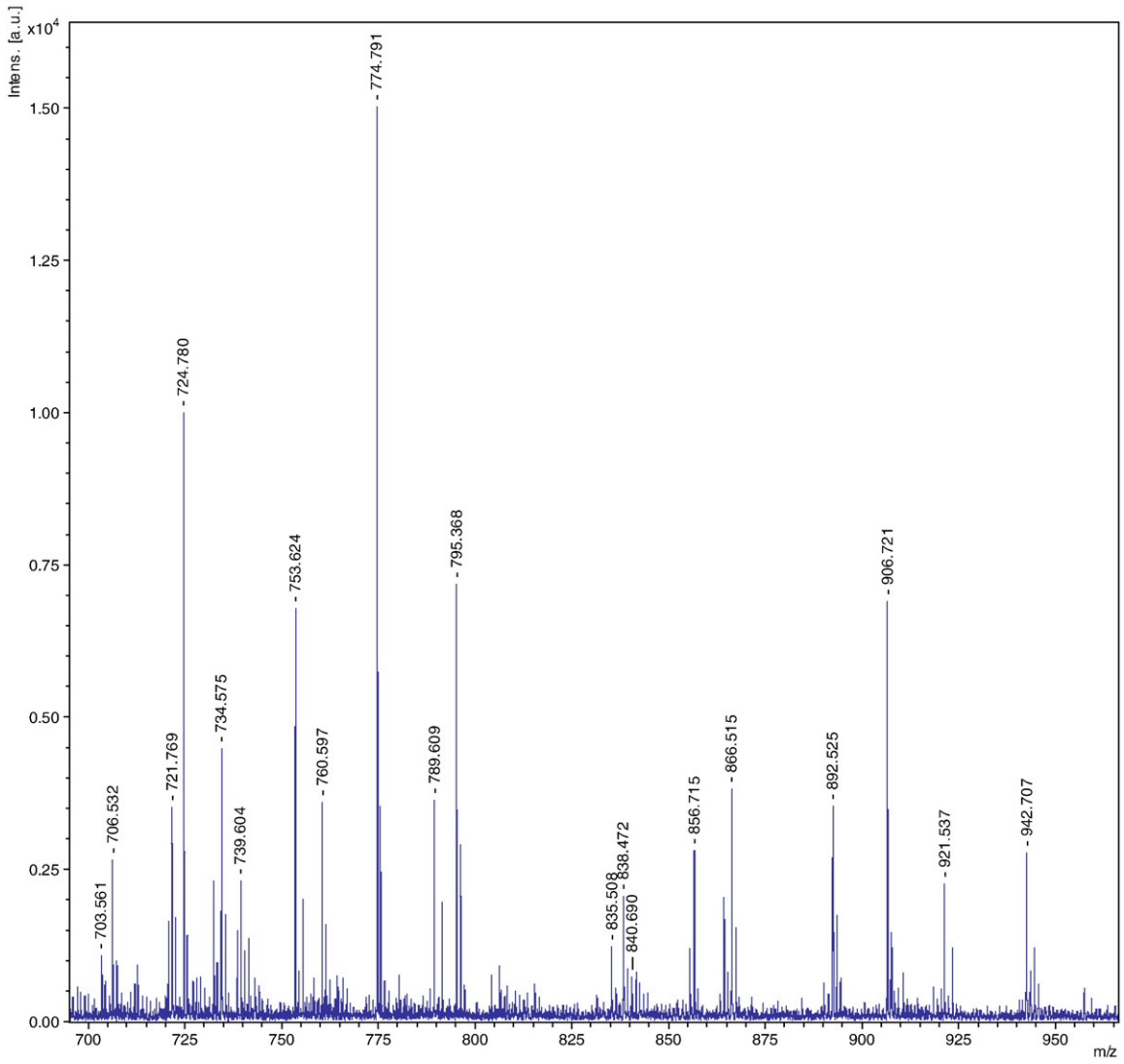

Fig. 6. MALDI-TOF MS spectra (positive ion reflector mode) of lipids extracted from porcine lenses. The matrix comprised p-nitroanaline in chloroform/methanol with crystals of cesium chloride.

Table 1
MALDI-TOF MS (positive ionization) analysis of porcine lens lipids. The number given in parenthesis is the total number of carbon atoms and the total number of double bonds.

Peak position (m/z)	Peak assignment
703.56	[SM(16:0)+H]+
706.532	[PC(30:0)+H]+
721.769	Matrix
724.78	Matrix
734.575	[PC(32:0)+H]+
738.594	Matrix
753.624	Matrix
760.597	[PC(34:1)+H]+
774.791	Matrix
789.609	Matrix
791.606	Matrix
795.368	Matrix
835.508	[SM(16:0)+Cs]+
838.472	[PC(30:0)+Cs]+
856.715	Matrix
864.493	[PC(32:1)+Cs]+
866.515	[PC(32:0)+Cs]+
892.525	[PC(34:1)+Cs]+
892.705	Matrix
906.721	Matrix
921.537	[DHSM(22:0)+Cs]+
942.707	[PC(38:4)+Cs]+

structural transition in both intact lenses and lipids extracted from lenses derived from a phase transition of lens sphingomyelins. Intriguingly, a similar phenomenon was also observed in artificial lipid membranes composed of eggSM and cholesterol which also showed a peak-like increase in the lifetime of o-Ps at approximately the same temperature as in lens lipid samples. This behavior in o-Ps lifetime can be explained by melting of a eggSM-rich gel-like phase in an otherwise cholesterol-rich liquid-ordered phase, in keeping with recent results from Quinn and Wolf [31,32]. Yet, the impact of lens proteins or microscopic changes in the lens cannot be excluded. However, extrinsic and intrinsic proteins have shown not to influence bovine lipid order [33] supporting our finding that the anomalous behavior of o-Ps in intact lenses may arise from structural changes in the lens lipids.

Finally, the feasibility of PALS to detect phase transitions in eggSM was examined. These results convincingly show, in accordance with our previous results [21] that during the main phase transition of eggSM bilayers, an increase in the lifetime of o-Ps is seen (Fig. 3 insert).

We used MALDI-TOF MS to identify the major lipid components of porcine lens. The results are in good accordance with previous results [25–27]. In essence we found that the major phospholipid components are long chain PCs and SMs. In our study cholesterol was not

detected due to the setup of our system. The long chain PCs and SMs are likely to cause ordering of the lens fiber membranes and allow for more interactions between the phospholipids and cholesterol [34]. Consequently, the lens fiber cells are not renewed, these cells have to show high resistance to physical stress. It is thus also emphasized in the composition of these cell membranes. Furthermore, the low abundance of unsaturated acyl chains hinders the formation of free radicals upon oxidation.

Previous studies using small-angle x-ray diffraction detected an irreversible phase transition at 50 °C in whole eyes, which originated from structural changes in α-crystallin [35]. In this paper we show that the whole lens also displays another structural transition at 35.5 °C, which intriguingly is very close to the body temperature of porcine (39 °C). We believe that this transition is related to structural changes in the lens fiber cell membranes possibly indicating a gel-to-fluid-like transition in nano-scale in the lipid regions. Thus at body temperature the lens fiber cell membranes would be fluid or liquid-ordered like. Yet, even very small changes to the lipid composition (as has been shown in cataractous vs. clear lenses [15]) could drive the lens membranes to a more gel-like state which in turn could have a profound effect on the function of the lens proteins and to the formation of cataract.

Due to non-negligible scatter in the positron data it is worth considering the origin of the relevant uncertainties in the positron experiments. The lifetime components in biomolecular matter are wide continuous distributions of lifetimes, in contrast to condensed matter where the lifetime distribution is significantly narrower. In addition, as the amount of water in the measured samples is high, it is impossible to separate the o-Ps lifetime in water from the lifetime in the biomaterial itself with the present experimental setup where the measurement time is optimized at the cost of detector resolution. These uncertainties, even if they produce scatter in the determination of the lifetime components from the data, are systematic and independent of, e.g., the measurement temperature, and hence changes in the positron data can be reliably deducted to originate from the biomaterial itself.

It would obviously be beneficial if the time needed for the positron experiments could be reduced. It is however, a difficult task, as reasonable statistics must be obtained for reliable analysis on the lifetime spectra. Changing the geometry of the setup can be used to increase the counting rate for the detectors as gamma flux is related to the inverse square of the range to the source (sample), but when heat bath type temperature control is needed, major improvements to the present geometry are not possible. The benefit in increasing the positron activity and thus reducing the time to gather a sufficient amount of lifetime events is also limited, as the signal-to-noise ratio decreases together with the activity of the positron source and reduces the reliability of the analysis.

A partial improvement to the above-mentioned limitations can be obtained by combining the high efficiency of large scintillation heads (SHs) to the high resolution of smaller SHs by using two detector pairs simultaneously. The principle is presented more thoroughly in our earlier paper [23], but the main idea is to perform the scanning of the temperature range normally with observing the possible changes from the lifetime data obtained with the large SH's, similarly as in this work. In the temperature range where no changes are observed the lifetime events measured with the small SH's can be summed together to provide better statistics and gain more precise information on the lifetime components, most importantly separation of the water component from the biomaterial component, as shown in Fig 1. This kind of a 4-detector positron lifetime spectrometer is currently being developed.

Based on our results, we believe that PALS is a viable method for gaining additional information on biological tissues, especially since it enables non-destructive studies on intact sample tissues, not only on extracted compounds of tissues. When combined with atomistic simulations for detailed considerations of free volume pockets in biomolecular simulations [36–38], PALS can provide major added value to better understand both structural and dynamical features in biological matter.

Acknowledgements

This work was supported by funding from the Sigrid Juselius Foundation (JMH), the Finnish Cultural Foundation (JMH), the Finnish Eye Foundation (JMH), and the Academy of Finland (PS, FT, SW, and IV).

References

[1] T.E. Merchant, J.H. Lass, P. Meneses, J.V. Greiner, T. Glonek, Human crystalline lens phospholipid analysis with age, Invest. Ophthalmol. Vis. Sci. 32 (1991) 549–555.
[2] D. Borchman, W.C. Byrdwell, M.C. Yappert, Regional and age-dependent differences in the phospholipid composition of human lens membranes, Invest. Ophthalmol. Vis. Sci. 35 (1994) 3938–3942.
[3] L.K. Li, L. So, A. Spector, Age-dependent changes in the distribution and concentration of human lens cholesterol and phospholipids, Biochim. Biophys. Acta. 917 (1987) 112–120.
[4] C.R. Fleschner, R.J. Cenedella, Lipid composition of lens plasma membrane fractions enriched in fiber junctions, J. Lipid Res. 32 (1991) 45–53.
[5] L.K. Van, R.M. Duvoisin, H. Engelhardt, M. Wiedmann, A function for lipoxygenase in programmed organelle degradation, Nature 395 (1998) 392–395.
[6] U.P. Andley, Crystallins in the eye: function and pathology, Prog. Retin. Eye Res. 26 (2007) 78–98.
[7] J.P. Brady, D. Garland, Y. Duglas-Tabor, W.G. Robison Jr., A. Groome, E.F. Wawrousek, Targeted disruption of the mouse alpha A-crystallin gene induces cataract and cytoplasmic inclusion bodies containing the small heat shock protein alpha B-crystallin, Proc. Natl. Acad. Sci. U. S. A. 94 (1997) 884–889.
[8] S.M. Mulders, G.M. Preston, P.M. Deen, W.B. Guggino, C.H. van Os, P. Agre, Water channel properties of major intrinsic protein of lens, J. Biol. Chem. 270 (1995) 9010–9016.
[9] W.C. Byrdwell, D. Borchman, R.A. Porter, K.G. Taylor, M.C. Yappert, Separation and characterization of the unknown phospholipid in human lens membranes, Invest Ophthalmol. Vis. Sci. 35 (1994) 4333–4343.
[10] H. Bloemendal, A. Zweers, F. Vermorken, I. Dunia, E.L. Benedetti, The plasma membranes of eye lens fibres. Biochemical and structural characterization, Cell Differ. 1 (1972) 91–106.
[11] D. Borchman, N.A. Delamere, L.A. McCauley, C.A. Paterson, Studies on the distribution of cholesterol, phospholipid, and protein in the human and bovine lens, Lens Eye Toxic. Res. 6 (1989) 703–724.
[12] R.M. Epand, Cholesterol in bilayers of sphingomyelin or dihydrosphingomyelin at concentrations found in ocular lens membranes, Biophys. J. 84 (2003) 3102–3110.
[13] M. Kuikka, B. Ramstedt, H. Ohvo-Rekila, J. Tuuf, J.P. Slotte, Membrane properties of D-erythro-N-acyl sphingomyelins and their corresponding dihydro species, Biophys. J. 80 (2001) 2327–2337.
[14] D. Borchman, M.C. Yappert, P. Herrell, Structural characterization of human lens membrane lipid by infrared spectroscopy, Invest. Ophthalmol. Vis. Sci. 32 (1991) 2404–2416.
[15] D. Borchman, O.P. Lamba, M.C. Yappert, Structural characterization of lipid membranes from clear and cataractous human lenses, Exp. Eye Res. 57 (1993) 199–208.
[16] O.E. Mogensen, Positron Annihilation in Chemistry, Springer-Verlag, Heidelberg, 1995.
[17] Y.C. Jean, P.E. Mallon, D.M. Schrader, Principles and Application of Positron and Positronium Chemistry, World Scientific, Singapore, 2003.
[18] R.A. Pethrick, Positron annihilation — a probe for nanoscale voids and free volume? Prog. Polym. Sci. 22 (1997) 1–47.
[19] M.E. Phelps, E.J. Hoffman, N.A. Mullani, M.M. Ter-Pogossian, Application of annihilation coincidence detection to transaxial reconstruction tomography, J. Nucl. Med. 16 (1975) 210–224.
[20] M.E. Phelps, PET — Molecular Imaging and Its Biological Applications, Springer Verlag, Berlin, 2004.
[21] P. Sane, E. Salonen, E. Falck, J. Repakova, F. Tuomisto, J.M. Holopainen, I. Vattulainen, Probing biomembranes with positrons, J. Phys. Chem. B. 113 (2009) 1810–1812.
[22] J. Folch, M. Lees, G.H. Sloane Stanley, A simple method for the isolation and purification of total lipides from animal tissues, J. Biol. Chem. 226 (1957) 497–509.
[23] P. Sane, S. Kilpeläinen, F. Tuomisto, 4-channel digital positron lifetime spectrometer for studying biological samples, Mater. Sci. Forum. 607 (2009) 254–256.
[24] European Union Council Directive 96/29/EURATOM, 1996.
[25] R. Estrada, D. Borchman, J. Reddan, A. Hitt, M.C. Yappert, In vitro and in situ tracking of choline-phospholipid biogenesis by MALDI TOF-MS, Anal. Chem. 78 (2006) 1174–1180.
[26] R. Estrada, M.C. Yappert, Regional phospholipid analysis of porcine lens membranes by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, J. Mass Spectrom. 39 (2004) 1531–1540.
[27] R. Estrada, M.C. Yappert, lternative approaches for the detection of various phospholipid classes by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry, J. Mass Spectrom. 39 (2004) 412–422.
[28] G.G. Shipley, L.S. Avecilla, D.M. Small, Phase behavior and structure of aqueous dispersions of sphingomyelin, J. Lipid Res. 15 (1974) 124–131.

[29] N.A. Delamere, C.A. Paterson, D. Borchman, K.L. King, S.A. Cawood, Calcium transport, Ca^{2+}-ATPase, and lipid order in rabbit ocular lens membranes, Am. J. Physiol. 260 (1991) C731–C737.
[30] T. Gonen, Y. Cheng, P. Sliz, Y. Hiroaki, Y. Fujiyoshi, S.C. Harrison, T. Walz, Lipid-protein interactions in double-layered two-dimensional AQP0 crystals, Nature 438 (2005) 633–638.
[31] C. Chachaty, C.D. Rainteau, C. Tessier, P.J. Quinn, C. Wolf, Building up of the liquid-ordered phase formed by sphingomyelin and cholesterol, Biophys J. 88 (2005) 4032–4044.
[32] P.J. Quinn, C. Wolf, Thermotropic and structural evaluation of the interaction of natural sphingomyelins and cholesterol, Biochim. Biophys. Acta. 1788 (2009) 1877–1889.
[33] H. Sato, D. Borchman, Y. Ozaki, O.P. Lamba, W.C. Byrdwell, M.C. Yappert, C.A. Paterson, Lipid-protein interactions in human and bovine lens membranes by Fourier transform Raman and infrared spectroscopies, Exp. Eye Res. 62 (1996) 47–53.
[34] J.M. Holopainen, A.J. Metso, J.P. Mattila, A. Jutila, P.K.J. Kinnunen, Evidence for the lack of a specific interaction between cholesterol and sphingomyelin, Biophys. J. 86 (2004) 1510–1520.
[35] J.W. Regini, J.G. Grossmann, M.R. Burgio, N.S. Malik, J.F. Koretz, S.A. Hodson, G.F. Elliott, Structural changes in alpha-crystallin and whole eye lens during heating, observed by low-angle X-ray diffraction, J. Mol. Biol. 336 (2004) 1185–1194.
[36] E. Falck, M. Patra, M. Karttunen, M.T. Hyvönen, I. Vattulainen, Lessons of slicing membranes: interplay of packing, free area, and lateral diffusion in phospholipid/cholesterol bilayers, Biophys. J. 87 (2004) 1076–1091.
[37] E. Falck, M. Patra, M. Karttunen, M.T. Hyvönen, I. Vattulainen, Impact of cholesterol on voids in phospholipid membranes, J. Chem. Phys. 121 (2004) 12676–12689.
[38] M. Kupiainen, E. Falck, S. Ollila, P. Niemelä, A.A. Gurtovenko, M.T. Hyvönen, M. Patra, M. Karttunen, I. Vattulainen, Free volume properties of sphingomyelin, DMPC, DPPC, PLPC bilayers, J. Comput. Theor. Nanosci. 2 (2005) 401–413.